British Journal of Pharmacology (1998) 123, 1621–1630

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Endothelin receptors mediating contraction of rat and human pulmonary resistance arteries: effect of chronic hypoxia in the rat

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1 We examined the endothelin (ET) receptors mediating contractions to ET-1, ET-3 and sarafotoxin S6c (SX6c) in rat pulmonary resistance arteries by use of peptide and non-peptide ET receptor antagonists. Changes induced by pulmonary hypertension were examined in the chronically hypoxic rat. The effect of the mixed ET_A/ET_B receptor antagonist SB 209670 on endothelin-mediated contraction was also examined in human pulmonary resistance arteries.

2 In rat vessels, the order of potency for the endothelin agonists was SX6c = ET-3 > ET-1 (pEC_{50} values in control rats: 9.12 ± 0.10, 8.76 ± 0.14 and 8.12 ± 0.04, respectively). Maximum contractions induced by ET-3 and ET-1 were increased in vessels from chronically hypoxic rats.

3 The ET_A receptor antagonist FR 139317 (1 μM) had no effect on the potency of ET-1 in any vessel studied but abolished the increased response to ET-1 in the chronically hypoxic vessels. The ET_A receptor antagonist BMS 182874 (1 μM) increased the potency of ET-1 in control rat vessels without effecting potency in the pulmonary hypertensive rat vessels.

4 Bosentan (non-peptide mixed ET_A/ET_B receptor antagonist) increased the potency of ET-1 in control rat vessels but was without effect in the pulmonary hypertensive rat vessels. Bosentan (1 μM) inhibited responses to SX6c in control and chronically hypoxic rat vessels with pK_b values of 5.84 and 6.11, respectively. The ET_B receptor antagonist BQ-788 (1 μM) did not inhibit responses to ET-1 in any vessel tested but did inhibit responses to both SX6c and ET-3 (pK_b values in control and chronically hypoxic rat vessels respectively: SX6c 7.15 and 7.22; ET-3: 6.68 and 6.89). BQ-788 (1 μM) added with BMS 182874 (10 μM) did not inhibit responses to ET-1 in control vessels but caused a significant inhibition of responses to ET-1 in chronically hypoxic preparations.

5 SB 209670 inhibited responses to ET-1 in both control and chronically hypoxic vessels with pK_b values of 7.36 and 7.39, respectively. SB 209670 (0.1 and 1 μM) virtually abolished responses to ET-1 in the human pulmonary resistance artery.

6 In conclusion, in rat pulmonary resistance arteries, vasoconstrictions induced by ET-1, SX6c and ET-3 are mediated predominantly by activation of an ET_B-like receptor. However, lack of effect of some antagonists on ET-1 induced vasoconstriction suggests that ET-1 stimulates an atypical ET_B receptor. The increase in potency of ET-1 in the presence of some antagonists suggests the presence of an inhibitory ET_A-like receptor. The influence of this is reduced, or absent, in the chronically hypoxic rats. Increased responses to ET-1 are observed in the chronically hypoxic rat and may be mediated by increased activation of ET_A receptors. SB 209670 is unique in its potency against responses to ET-1 in both control and chronically hypoxic rats, as well as human, isolated pulmonary resistance arteries.

Keywords: Endothelin receptors; pulmonary arteries; pulmonary hypertension; SB 209670

Introduction

The family of endothelin peptides are known to have diverse roles in many physiological systems (Rubanyi & Polokoff, 1994). In the pulmonary circulation endothelins have been shown to produce both vasoconstriction and vasodilatation depending on the species/peptide studied and the experimental conditions (Barnes & Liu, 1995). These opposing effects can be explained in part by the physiological effects of the two main endothelin receptor subtypes. The ET_A receptor demonstrates selectivity for endothelin-1 (ET-1) over endothelin-3 (ET-3) whereas the ET_B receptor is non isopeptide selective (Arai *et al*., 1990, Sakurai *et al*., 1990). Although both have been shown to mediate vasoconstriction, the ET_B receptor also mediates vasodilatation through endothelial release of nitric oxide and prostanoids (Masaki *et al*., 1991). There is also evidence for at least two novel human ET_B receptor splice variants (Shyamala *et al*., 1994; Elshourbagy *et al*., 1996).

We have previously shown that the receptor subtypes mediating ET-1 vasoconstriction in isolated pulmonary arteries of the rat varies depending on the size and/or location of the artery under study. ET_A receptors mediate vasoconstriction in the larger elastic pulmonary arteries whilst the ET_B receptor subtype mediates vasoconstriction in the pulmonary resistance arteries (MacLean *et al*., 1994). A similar situation is thought to occur in piglet pulmonary arteries (Perrault & Baribeau, 1995). However, in the rabbit large pulmonary artery ET-1 mediated vasoconstriction is almost entirely ET_B receptor mediated (LaDouceur *et al*., 1993). Regional heterogeneity has also been described in the human coronary circulation (Godfraind, 1993).

However, preliminary data from studies on rat pulmonary resistance arteries and studies in human and rabbit pulmonary resistance have indicated that the receptor involved in ET-1-induced vasoconstriction in the pulmonary resistance artery is not a typical ET_A or ET_B receptor. ET-1-mediated vasocon-

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striction is resistant to ET_A- and ET_B-antagonists and combinations of these (McCulloch & MacLean, 1995; McCulloch *et al*., 1996; Docherty & MacLean, 1998). Here we investigate further the receptors mediators the effects of the endothelin receptor agonists ET-1, ET-3 and the ET_B-selective agonist sarafotoxin S6c (SX6c) in the rat pulmonary resistance artery.

There is increased pulmonary gene expression for ET-1 after chronic hypoxia in rats (Elton *et al*., 1992). However, it is still unclear if ET-1 is a mediator or modulator of pulmonary hypertension (Stewart *et al*., 1991). We have previously shown that responses to ET-1 are altered in rat conduit pulmonary arteries from chronic hypoxic rats (MacLean *et al*., 1995). We therefore studied pulmonary resistance arteries from both control rats and rats subjected to fourteen days chronic hypoxia to assess any changes which may occur in the pulmonary hypertensive state. It remains unclear whether a selective ET_A or mixed ET_A/ET_B receptor antagonist will be more effective in the treatment of this disease. We wished, therefore, to compare the potency of SB 209670 against ET-1 with that of other endothelin antagonists.

We recently published evidence for a heterogeneous population of endothelin receptors in human pulmonary resistance arteries (McCulloch *et al*., 1996). At the time this study was conducted SB 209670 was not available, although we suggested that such an antagonist might be most effective against responses to ET-1 in these vessels. The results of this study indicated that the effect of SB 209670 was of particular interest, and so we studied the effect of SB 209670 on responses to ET-1 in human pulmonary resistance arteries.

Methods

Chronically hypoxic rats

Male Wistar rats of 28–30 days (at start of experiment) were placed in a specially designed perspex hypobaric chamber (Royal Hallamshire Hospital Sheffield). This was depressurized, over two days, to 550 mbar (equivalent PO_2 of $\cong$110 mmHg). the temperature of the chamber was maintained at 21–22°C and the chamber was ventilated with air at approximately 45 l min^{-1}. Animals were maintained in these hypoxic/hypobaric conditions for two weeks. Aged-matched controls were maintained in room air under normal atmospheric pressures.

Human pulmonary arteries

Pulmonary arteries (150–200 μm, i.d.) were dissected from grossly normal sections of human lung removed from post-operative bronchial carcinoma tissue. Samples were refrigerated in fresh Krebs solution and were collected for study no longer than 12 h after the operation.

Experimental procedure

Rats were killed by an overdose of sodium pentobarbitone and the heart and lungs removed and placed in cold Krebs. The right ventricle of the heart was carefully dissected free of the septum and left ventricle and these were blotted lightly and weighed. Pulmonary hypertension was assessed by measuring the ratio of right ventricular (RV)/total ventricular (TV) weight. This is a well established index of the degree of pulmonary hypertension in rats (Hunter *et al*., 1974; Leach *et al*., 1977).

Intralobar resistance arteries ($\sim$150 μm i.d.) were carefully dissected out and cleaned of surrounding parenchyma. Control and chronically hypoxic rat and human vessel pairs were then mounted as ring preparations (2 mm long) in the bath of a resistance vessel myograph. All vessels were bathed in Krebs solution at 37°C and bubbled with 16% O_2/5% CO_2 balance N_2. Vessels were subjected to tension to give transmural pressures equivalent to 15.9$\pm$0.4 mmHg for control rat and human vessels, and 35.6$\pm$0.3 for the chronically hypoxic group, which are approximately the pressures of pulmonary arteries and arterioles of these animals *in vivo* (Herget *et al*., 1978).

After one hour equilibration, vessels were contracted with 50 mM KCl twice. The integrity of the vascular endothelium in rats was then determined by examining for relaxations to 1 μM ACh in the presence of raised vascular tone (preconstricted with 10 μM 5-HT). Preparations were then subjected to one of the following protocols. (A) Forty five minute equilibration period followed by a cumulative concentration-response curve (CCRC) to either ET-1, ET-3 or sarafotoxin S6c (SX6c) (0.01 pM to 0.3 μM); (B) 45 min incubation with one concentration of the antagonist under study followed by CCRC to selected agonist.

Control responses to ET-1 were carried out, whenever possible, in vessels from each lung studied. In each table, responses to agonists in the presence of antagonists are compared with agonist responses determined from the same lungs. Concentrations of antagonists studied were chosen from their calculated pA_2/pK_b values in other vascular preparations. The antagonists studied were FR139317 (pA_2 vs ET-1 in rabbit aorta: 7.2, Sogabe *et al*., 1993); BMS 182874 (pA_2 vs ET-1 in rabbit coronary artery: 6.5, Stein *et al*., 1994); BQ-788 (pA_2 vs ET-1 in porcine coronary arteries: 7.4, Ishikawa *et al*., 1994) and SB 209670 (pK_b vs ET-1 in rabbit pulmonary artery: 6.2, Ohlstein *et al*., 1994a,b).

Drugs and solutions

The composition of the Krebs-bicarbonate saline (pH 7.4) was as follows (in mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, $CaCl_2$ 2.5 and glucose 11. The following drugs were used: endothelin-1 (Thistle, Glasgow, U.K.), endothelin-3 (Peninsula Laboratories), sarafotoxin S6c (Sigma), BQ-788 (N-*cis*-2,6-dimethylpiperidinocarbonyl L-γ-MeLeu-D-Trp ($COOCH_3$)-D-Nle; Peptide International), FR139317 (N-CO-L-Leu-D-1-Me-Trp-D-3(2-pyridyl) Ala-OH; Neosystems). BMS 182874 (5-dimethylamino)-*N*-(3,4-dimethyl-5-isoxzolyl)-1-napthalenesulphonamide) and SB 209670 ((+)-(1**S**, 2**R**,3**S**)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)5-(prop-1-yloxy)indane-2-carboxylic acid; gift from SmithKline Beecham Pharmaceuticals). Bosentan (4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzene sulphonamide) was a welcome gift from Roche. Stock solutions of sarafotoxin S6c (100 μM) were prepared in 0.1% acetic acid and those to BQ-788 (1 mM) in 0.1% dimethylsulphoxide (DMSO). All other drugs and subsequent dilutions were prepared in distilled H_2O.

Data analysis

Results are expressed graphically as percentage of the reference contraction to the second application of 50 mM KCl. The pEC_{50} and pEC_{80} values (where appropriate) were calculated by computer interpolation from individual CCRCs and estimated pK_b values for antagonists were calculated. In this

respect we have made certain assumptions. We assumed that a maximum control response to ET-1 was achieved. Due to cost restraints, the maximum concentration of ET-1 used was 0.3 μM. We justify this on the grounds that we have previously determined that 1 μM ET-1 does not produce a significantly greater response (unpublished observation). We also assumed that, in the presence of the SB 209670, ET-1 produces the same maximum response. An increase in the maximum response to ET-1 in the presence of SB 209670, due to blockade of endothelial ET_B receptors, has been demonstrated in rabbit conduit pulmonary arteries (Ohlstein *et al*., 1994a). If this is also the case in the resistance arteries, we will have underestimated the pK_b values of SB 209670. However, there is no evidence for 'vasodilator' ET_B receptors in this preparation (MacLean & McCulloch, 1998). We also assumed that SX6c reached a maximum response before the 'desensitization' phenomenon observed.

Statistical comparisons of the means of groups of data were made by one way analysis of variance (ANOVA) followed by *ad hoc* Tukeys *post*-test where appropriate. $P<0.05$ was considered statistically significant. *n* in text refers to the number of animals studied. We chose not to curve fit these data as the concentration-response curve was not typically sigmoidal in nature and in the case of SX6c, there was a bell-shaped component to the curve. We are not aware of a widely available programme which can accommodate these curve forms.

Results

Rats exposed to 14 days hypobaric hypoxia showed significant right ventricular hypertrophy compared to aged-matched control animals. RV/TV ratio was 0.381 ± 0.01 for control animals and 0.625 ± 0.02 for chronically hypoxic animals ($n=8$; $P<0.001$) indicating the development of pulmonary hypertension in chronically hypoxic animals.

KCl (50 mM)-induced contractions were of the same magnitude in both control and chronically hypoxic pulmonary resistance arteries, being 270 ± 24 and 294 ± 24 mg wt tension, respectively ($n=20$ in both control and chronically hypoxic groups). Both control and chronically hypoxic vessels exhibited significant vasodilatation to ACh, indicating preservation of endothelial function.

Responses to ET-1, ET-3 and SX6c

In control rats, all three peptides produced vasoconstrictor responses, the order of potency being SX6c = ET-3 > ET-1 (Figure 1a, Table 1). The pEC_{50} value for ET-1 in this cohort of animals was 8.12 ± 0.04 ($n=8$). SX6c was 10 fold more potent than ET-1 in this preparation. At the pEC_{80} level the potency of SX6c was significantly greater than that to ET-3 (pEC_{80} values being ET-3 control 7.89 ± 0.08, and SX6c control 8.51 ± 0.12; $P<0.01$, Student's unpaired *t* test). The maximum contractile response achieved to ET-3 in control vessels was significantly less than that achieved to ET-1 ($P<0.05$) but not significantly different from SX6c, although there was a comparatively wide variation in maximum response to SX6c (Figure 1a). There was a 'drop-off' in the response to SX6c at ~0.01 μM, a phenomenon which we have described and discussed previously (MacLean *et al*., 1994; MacLean & McCulloch, 1998).

In chronically hypoxic rats the order of potency was SX6c = ET-3 > ET-1 (Figure 1b, Table 1). The pEC_{50} value for ET-1 in this cohort of animals was 8.25 ± 0.08 ($n=6$). SX6c was over 10 fold more potent than ET-1 in this preparation whilst ET-1 produced a significantly greater maximum contraction than SX6c ($P<0.01$, Figure 1b). ET-3 was 5 fold more potent than ET-1 and whilst the maximum contractile response to ET-3 was similar to that of ET-1, it was significantly greater than SX6c ($P<0.05$, Figure 1b). Again, at the pEC_{80} level the potency of SX6c was significantly greater than that of ET-3 (pEC_{80} values being ET-3 hypoxic 7.84 ± 0.16, and SX6c hypoxic 8.64 ± 0.08; $P<0.05$).

Maximum responses to both ET-1 and ET-3 were increased by hypoxia (ET-1: 186 ± 18 vs 292 ± 32, $n=8$, $P<0.001$; ET-3: 122 ± 9 vs 209 ± 15, $n=8$, $P<0.05$). The maximum response to SX6c was not significantly altered by hypoxia at any concentration and there was no change in the pEC_{50} values for any agonist (Table 1).

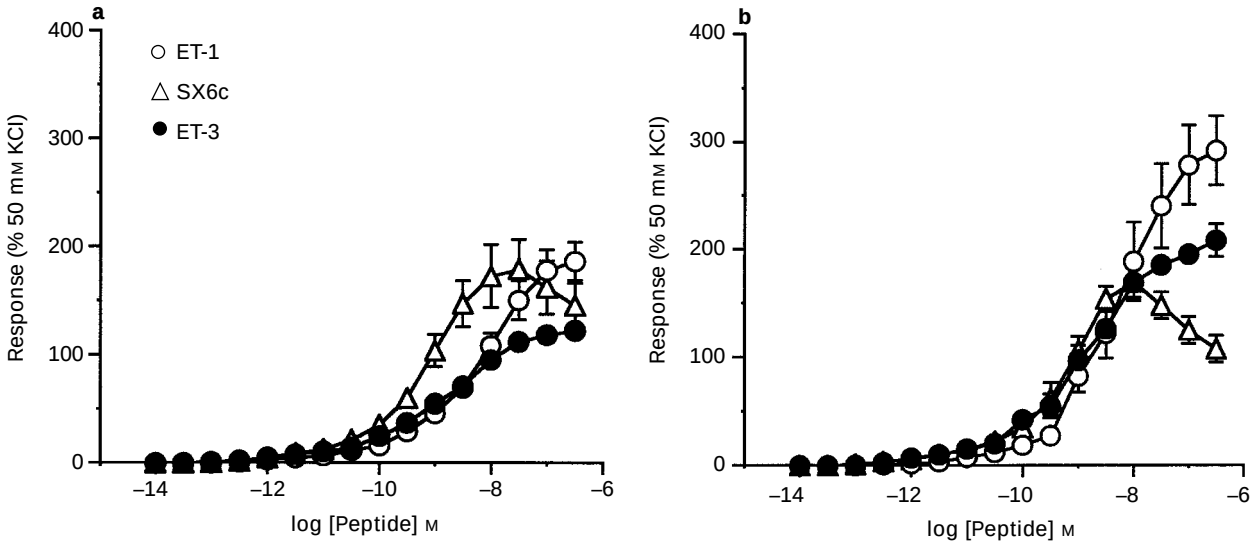

Figure 1 Endothelin-1 (ET-1), endothelin-3 (ET-3) and sarafotoxin S6c (SX6c)-induced vasoconstriction in control rat (a) and chronically hypoxic rat (b) pulmonary resistance arteries. ET-1 ($n=8$ (a); 6/6 (b)), SX6c ($n=8$(a); 8 (b)), and ET-3 ($n=4$ (a); 4 (b)). Data are expressed as percentage of reference contraction to 50 mM KCl in each vessel. *n* = number of animals. Each point represents the mean and vertical lines show s.e.mean.

Effects of ET_A receptor antagonists on responses to ET-1

Incubation with all the antagonists used in these studies had no effect on baseline tension in control and chronically hypoxic rat preparations.

FR 139319 (1 μM) did not affect the potency of, or the maximum contractile response to, ET-1 in control preparations (Figure 2a, Table 1), but caused a significant decrease in the maximum contractile response to ET-1 in chronically hypoxic vessels ($P<0.05$, Figure 2b) reducing the maximum to that observed in the vessels removed from control rats. This decrease in the maximum contractile response occurred without an affect on the potency of ET-1 (Table 1).

Responses to ET-1 in chronically hypoxic pulmonary resistance arteries were unaltered in the presence of BMS 182874 (1 μM), but the antagonist caused a small though significant increase in ET-1 potency in control preparations (Figure 2a and Table 1). This increase in potency occurred without an affect on the maximum contractile response to ET-1 (Figure 2a).

Effect of bosentan on responses to ET-1

Bosentan (1 μM) caused a small but significant increase in ET-1 potency in control rat preparations without significantly changing the maximum contractile response to ET-1 (Figure 3a, Table 1). Bosentan had no significant effect on responses to ET-1 in chronically hypoxic preparations (Figure 3b, Table 1).

Effect of BQ-788 and SB 209670 on responses to ET-1

BQ-788 (1 μM) alone had no effect on responses to ET-1 in control or chronically hypoxic preparations (Figure 4a and b,

Table 1 Potency of endothelin (ET) peptides and effect of antagonist on reponses to ET-1, ET-3 and sarafotoxin S6c (SX6c) in rat pulmonary resistance arteries

	Controls		*Hypoxic*	
	pEC_{50}	n	pEC_{50}	n
ET-1	8.09±0.04	26	8.18±0.10	23
SX6c	9.12±0.10[a]	8	9.21±0.13[b]	8
ET-3	8.76±0.14[c]	4	8.82±0.15[d]	4
ET-1 + FR139317 (1 μM)	8.02±0.22	6	8.00±0.19	6
ET-1 + BMS 182874 (1 μM)	8.86±0.23**	5	8.31±0.09	5
ET-1 + bosentan (1 μM)	8.40±0.09**	4	8.15±0.05	4
ET-1 + BQ-788 (1 μM)	8.48±0.23	5	8.47±0.18	5
ET-1 + BQ-788 + BMS 182874 (10 μM)	8.10±0.09	7	7.91±0.07*	7
ET-1 + SB 209670 (10 nM)	7.78±0.19	9	8.11±0.19	4
ET-1 + SB 209670 (0.1 μM)	7.71±0.13*	4	7.56±0.16**	4
ET-1 + SB 209670 (1.0 μM)	7.16±0.07***	5	7.07±0.08***	4
SX6c + bosentan (0.1 μM)	9.54±0.58	4	9.36±0.45	4
SX6c + bosentan (1.0 μM)	8.79±0.09†	5	8.67±0.12†	5
SX6c + BQ-788 (1.0 μM)	7.85±0.08†††	4	7.79±0.07†††	4
ET-3 + BQ-788 (1.0 μM)	8.01±0.22[c]	3	7.78±0.33[e]	3

Data are shown as mean±s.e.mean; n = number of animals. Controls: vessels from control rats, hypoxic: vessels from rats subjected to chronic hypoxia for 14 days. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Tukeys *ad hoc post* test. The data sets compared were derived from preparations studied in common animals ($n=4-8$). [a]$P<0.001$: control ET-1 vs control SXS6c. [b]$P<0.001$: chronic hypoxic (CH) ET-1 vs CH SXS6c. [c]$P<0.001$: control ET-1 vs Control ET-3. [d]$P<0.01$: CH ET-1 vs CH ET-3. [e]$P<0.05$: ET-3 vs ET-3 (+antagonist). *$P<0.05$, **$P<0.01$, ***$P<0.001$: ET-1 vs ET-1 (+antagonist). †$P<0.05$, †††$P<0.001$: SX6c vs SX6c (+antagonist).

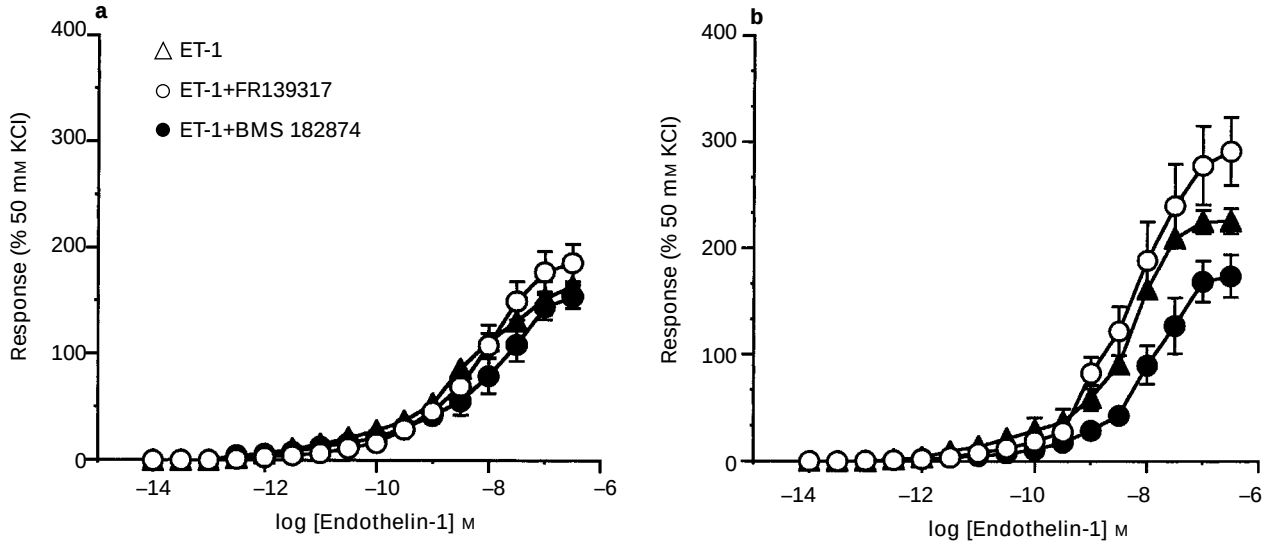

Figure 2 Effect of FR139317 and BMS 182874 on responses to endothelin-1 (ET-1) in rat pulmonary resistance arteries. Responses to ET-1 in control rat vessels (a) and chronically hypoxic rat vessels (b). ET-1 ($n=8$ (a and b)), ET-1 in the presence of 1 μM FR139317 ($n=6$ (a and b)), and ET-1 in presence of 1 μM BMS 182874 ($n=5$ (a and b)). Data are expressed as percentage of reference contraction to 50 mM KCl in each vessel. n = number of animals. Each point represents the mean and vertical lines show s.e.mean.

Table 1). The pEC_{50} values for ET-1 in these cohorts of animals was 8.12 ± 0.04 (controls, $n=9$) and 8.17 ± 0.09 (chronically hypoxic, $n=8$). However, a combination of BQ-788 (1 μM) and BMS 182874 (10 μM) caused a significant inhibition of response to ET-1 in chronically hypoxic preparations but not vessels from control rats (Figure 4a and b, Table 1).

SB 209670 inhibited responses to ET-1 in control and chronically hypoxic rat vessel preparations (Figure 5a and b, Table 1). The pEC_{50} values for ET-1 in these cohorts of animals ($n=9$) was 8.01 ± 0.05 (controls) and 8.11 ± 0.07 (chronically hypoxic). pK_b values for SB 209670 (0.1 μM) against ET-1 are shown in Table 2.

Effect of antagonists on SX6c responses

Bosentan (1 μM) inhibited responses to SX6c in both control and chronically hypoxic preparations (Figure 6a and b, Table 1). BQ-788 inhibited responses to SX6c in both control and chronically hypoxic vessels (Figure 7a and b, Table 1). The estimated pK_b values for bosentan and BQ-788 against SX6c-induced contractions are shown in Table 2.

Effect of BQ-788 on ET-3 responses

BQ-788 (1 μM) caused a significant inhibition of contraction to ET-3 in both control and chronically hypoxic rat vessels (Figure 8a and b, Table 1). The pK_b values for BQ-788 against ET-3 are presented in Table 2, which shows that pK_b values for BQ-788 against ET-3 were significantly less than values obtained for BQ-788 against SX6c.

Effect of SB 209670 on ET-1 responses in human pulmonary resistance arteries

SB 209670 was extremely potent against ET-1, causing an approximate 10–100 fold decrease in potency (Figure 9). The

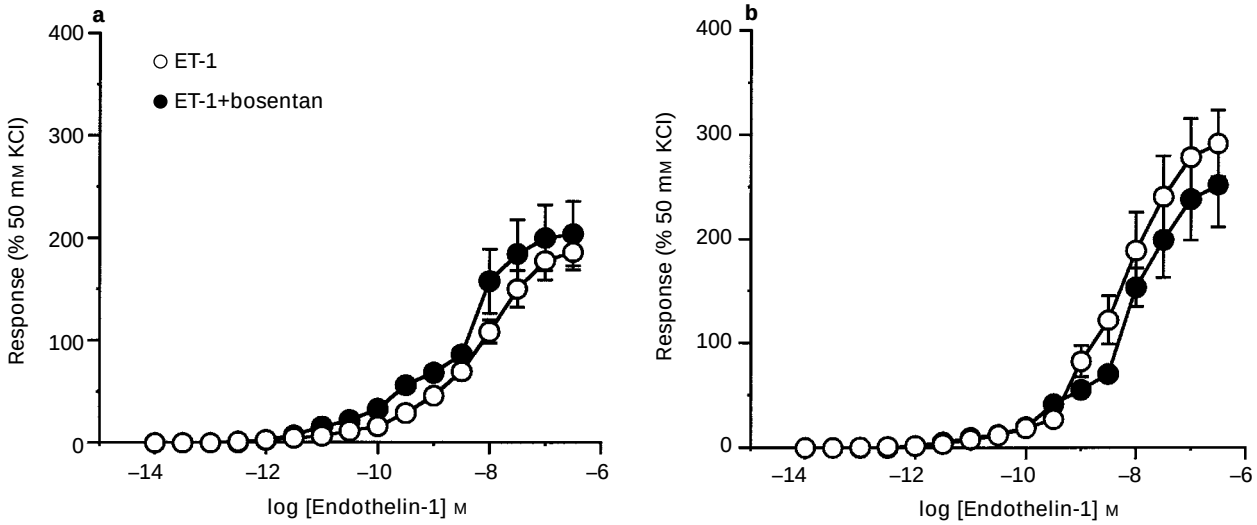

Figure 3 Effect of bosentan on responses to endothelin-1 (ET-1) in rat pulmonary resistance arteries. Responses to ET-1 in control rat vessels (a) and chronically hypoxic rat vessels (b). ET-1 ($n=8$) (a and b), ET-1 in the presence of 1 μM bosentan ($n=4$ (a and b)). Data are expressed as percentage of reference contraction to 50 mM KCl in each vessel. n=number of animals. Each point represents the mean and vertical lines show s.e.mean.

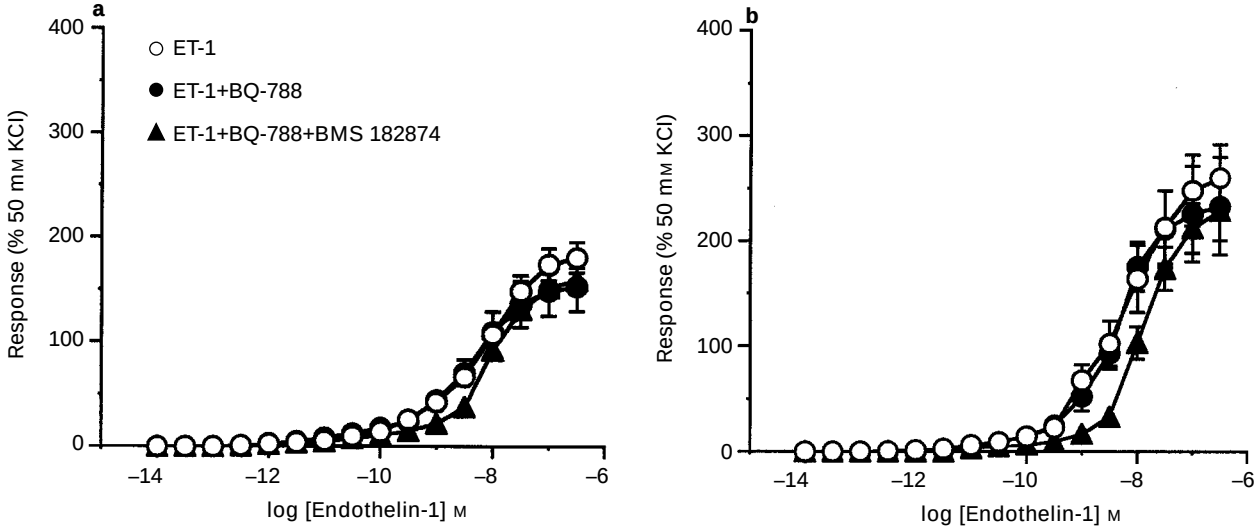

Figure 4 Effect of BQ-788 (1 μM), and a combination of BQ-788 (1 μM) + BMS 182874 (10 μM) on responses to endothelin-1 (ET-1) in rat pulmonary resistance arteries. Responses to ET-1 in control rat vessels (a) and chronically hypoxic rat vessels (b). ET-1 ($n=9$ (a) $n=8$ (b)), ET-1 in the presence of 1 μM BQ-788 ($n=5$ (a and b)), and ET-1 in presence of 1 μM BQ-788 and 10 μM BMS 182874 ($n=7$ (a and b)). Data are expressed as percentage of reference contraction to 50 mM KCl in each vessel. n=number of animals. Each point represents the mean and vertical lines show s.e.mean.

pEC_{50} value for ET-1 in the human vessels was 8.10 ± 0.20 ($n = 8$ lungs).

Discussion

The present study indicates that contractions mediated by ET-1, ET-3 and SX6c in the control rat pulmonary resistance artery are mediated via activation of the ET_B receptor. However, the endothelin agonist potencies and the inability of classical ET_A and ET_B selective antagonists to inhibit responses to ET-1 suggest that there is ET-1-mediated vasoconstriction via activation of an atypical endothelin receptor. SX6c and ET-3 are more potent than ET-1 in inducing vasoconstrictor responses in the rat pulmonary resistance arteries as we have previously demonstrated in human pulmonary resistance arteries (McCulloch *et al*., 1996). In addition, we show here that SX6c is more potent that ET-3 at the pEC_{80} level in the rat

Table 2 Estimated pK_b values for endothelin antagonists in rat pulmonary resistance arteries

	Control		*Hypoxic*	
Antagonist	pK_b	n	pK_b	n
SB 209670 (0.1 μM) vs endothelin-1	7.36 ± 0.04	4	7.39 ± 0.03	4
BQ-788 (1 μM) vs sarafotoxin S6c	7.15 ± 0.04	4	7.22 ± 0.02	4
BQ-788 (1 μM) vs endothelin-3	6.68 ± 0.07**	3	6.89 ± 0.17*	3
Bosentan (1 μM) vs sarafotoxin S6c	5.84 ± 0.13	5	6.11 ± 0.11	5

Data are shown as mean ± s.e.mean; n = number of animals. Control: vessels from control rats; hypoxic: vessels from rats subjected to chronic hypoxia for 14 days. Concentration of antagonist used to estimate pK_b is shown in parentheses. Statistical comparisons were made by Student's unpaired t test. $^*P < 0.05$, $^{**}P < 0.01$ BQ 788 (ET-3) vs BQ 788 (SX6c).

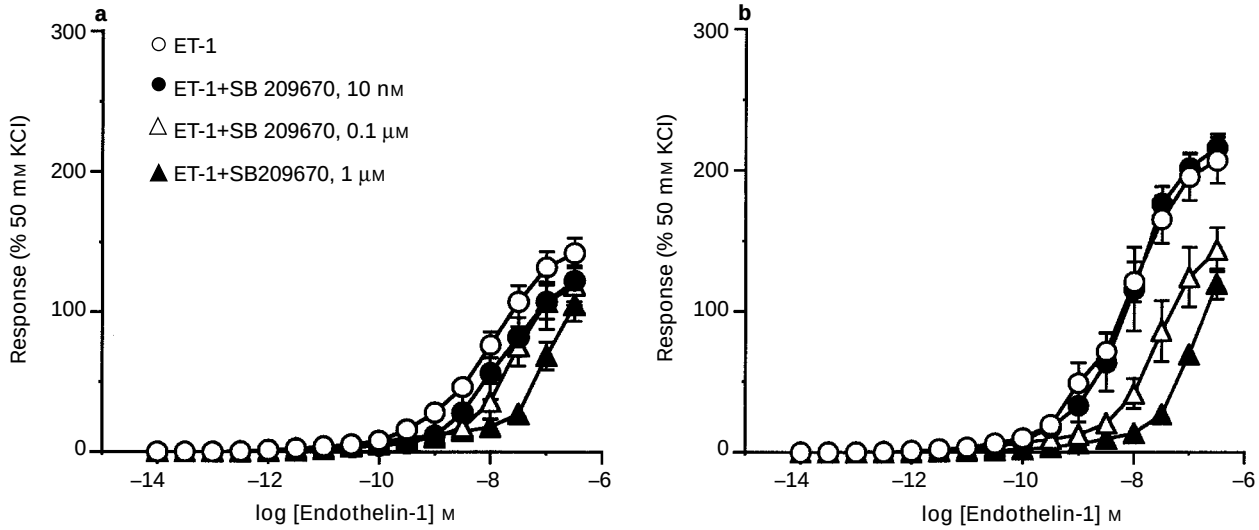

Figure 5 Effect of SB 209670 on responses to endothelin-1 (ET-1) in rat pulmonary resistance arteries. Responses to ET-1 in control rat vessels (a) and chronically hypoxic rat vessels (b). ET-1 ($n = 9$ (a and b)), ET-1 + 10 nM SB 209670 ($n = 4$ (a), $n = 9$ (b)), ET-1 + 0.1 μM SB 209670 ($n = 4$ (a and b)), and ET-1 in the presence of 1 μM SB 209670 ($n = 5$ (a and b)). Data are expressed as percentage of reference contraction to 50 mM KCl in each vessel. Each point represents the mean and vertical lines show s.e.mean.

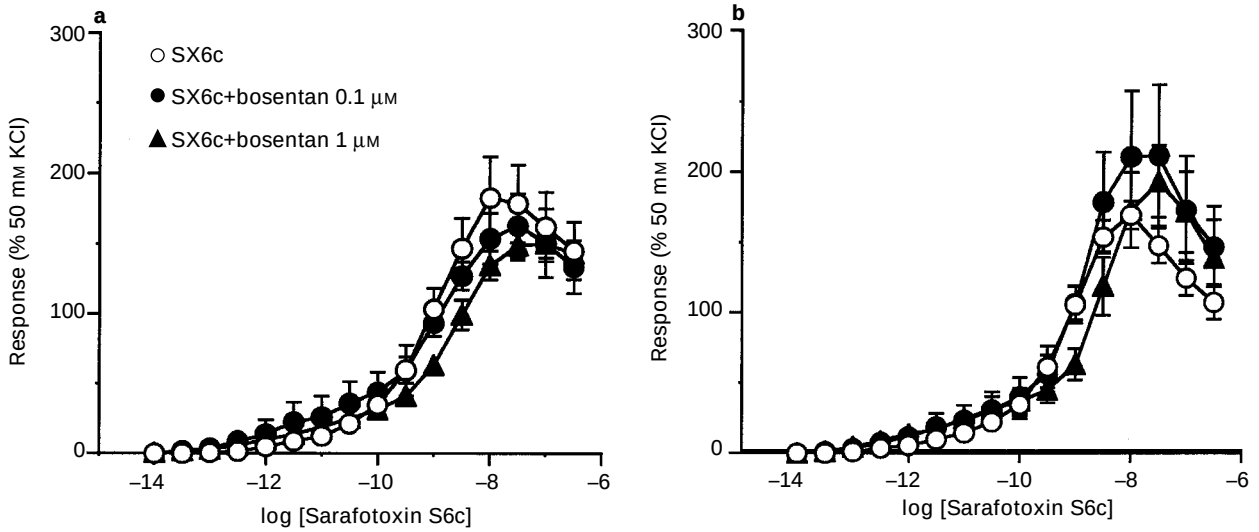

Figure 6 Effect of bosentan on responses to sarafotoxin S6c (SX6c) in rat pulmonary resistance arteries. Responses to SX6c in control rat vessels (a) and chronically hypoxic rat vessels (b). SX6c ($n = 8$ (a and b)), SX6c in the presence of 0.1 μM bosentan ($n = 4$ (a and b)), and Sx6c in presence of 1 μM bosentan ($n = 5$ (a and b)). Data are expressed as percentage of reference contraction to 50 mM KCl in each vessel. n = number of animals. Each point represents the mean and vertical lines show s.e.mean.

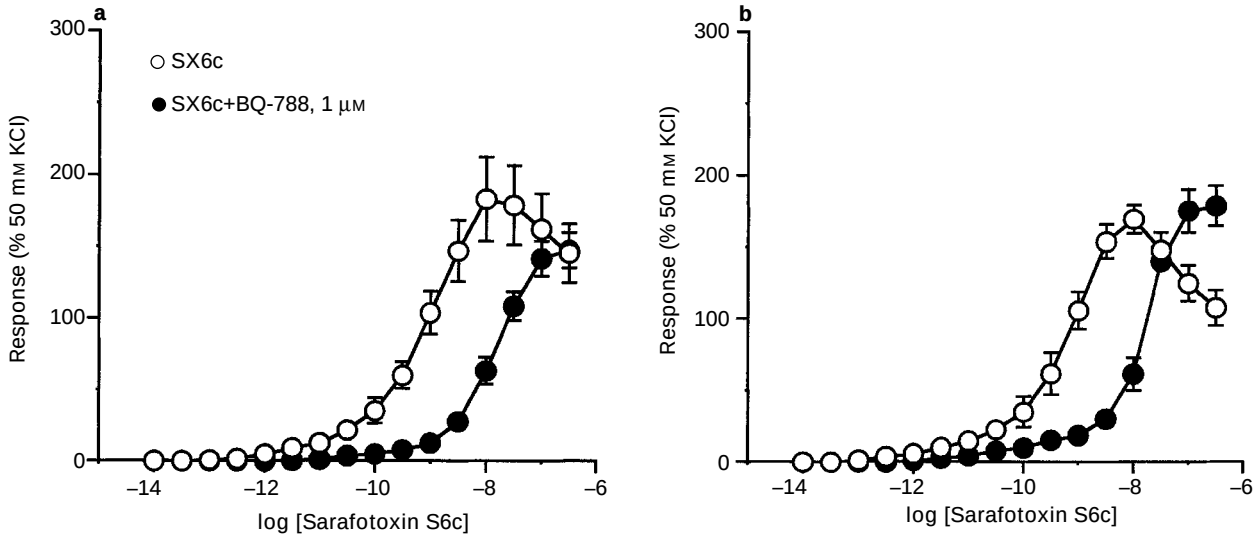

Figure 7 Effect of BQ-788 on responses to sarafotoxin S6c (SX6c) in rat pulmonary resistance arteries. Responses to SX6c in control rat vessels (a) and chronically hypoxic rat vessels (b). SX6c ($n=8$ (a and b)), SX6c in the presence of 1 μM BQ-788 ($n=4$ (a and b)). Data are expressed as percentage of reference contraction to 50 mM KCl in each vessel. n = number of animals. Each point represents the mean and vertical lines show s.e.mean.

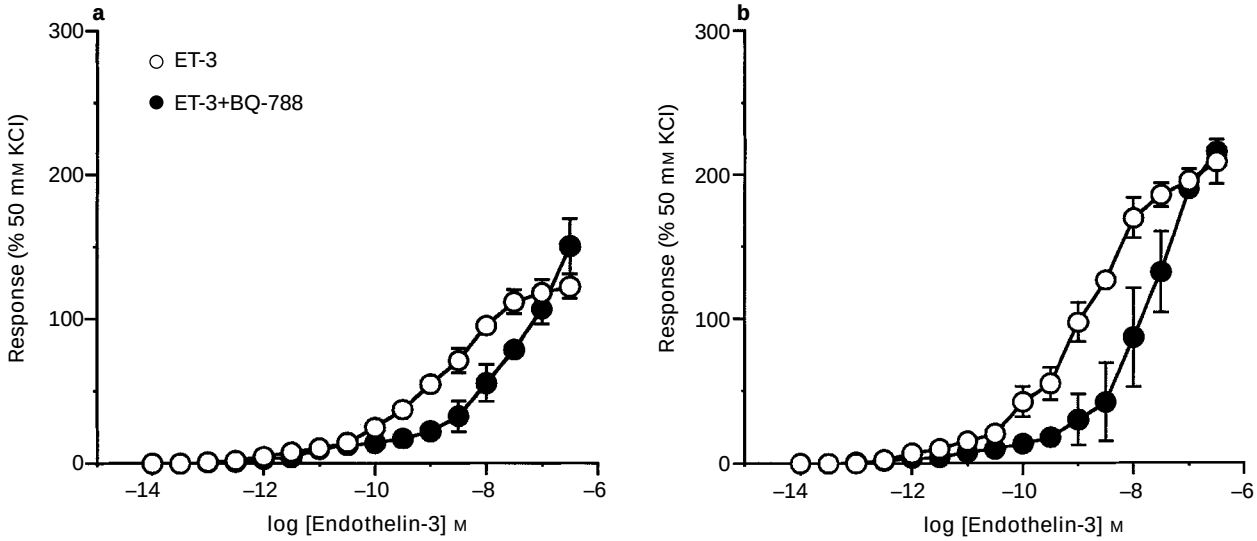

Figure 8 Effect of BQ-788 on responses to endothelin-3 (ET-3) in rat pulmonary resistance arteries. Responses to ET-3 in control rat vessels (a) and chronically hypoxic rat vessels (b). ET-3 ($n=4$ (a and b)), ET-3 + 1 μM BQ-788 ($n=3$ (a and b)). Data are expressed as percentage of reference contraction to 50 mM KCl in each vessel. n = number of animals. Each point represents the mean and vertical lines show s.e.mean.

vessels. In preparations with homogeneous population of 'classical' ET_B receptors, ET-1 and ET-3 are equipotent (Arai *et al*., 1990; Sakurai *et al*., 1990). This suggests that there may be a receptor present in rat pulmonary artery which shows selectivity for ET-3 over ET-1 and even greater selectivity for SX6c over ET-3 at higher concentrations. Such a receptor has been cloned from *Xenopus laevis* dermal mellanophores and has been denoted ET_C (Karne *et al*., 1993). However, a counterpart has yet to be cloned from a mammalian vascular preparation, although a receptor with similar pharmacological characteristics has been described in the rabbit lateral saphenous vein (Douglas *et al*., 1995). The potency of SX6c is greater than that of ET-1 in preparations such as the rabbit large pulmonary artery and bronchus, the guinea-pig bronchus and the rabbit pulmonary resistance artery (Hay *et al*., 1996; Hay & Luttman, 1997; Docherty & MacLean, 1998). However, in such preparations, responses to ET-1 are equipotent to ET-3, suggesting further diversity in the pharmacology of endothelin receptors.

Maximum contractile responses to ET-1 and ET-3 were significantly increased in chronically hypoxic pulmonary arteries compared to control preparations, whereas the maximum contractile response to SX6c was unchanged. Chronic hypoxia is known to cause pulmonary vascular remodelling, in which small pulmonary resistance arteries become muscularized (Hunter *et al*., 1974). If it were simply the case that all vasoconstrictor responses were increased due to pulmonary vascular remodelling, then we would have seen an increase in the maximum contraction to all vasoconstrictors. Indeed, we have shown that responses to 5-hydroxytryptamine are increased in pulmonary arteries from the chronically hypoxic rat (MacLean *et al*., 1996).

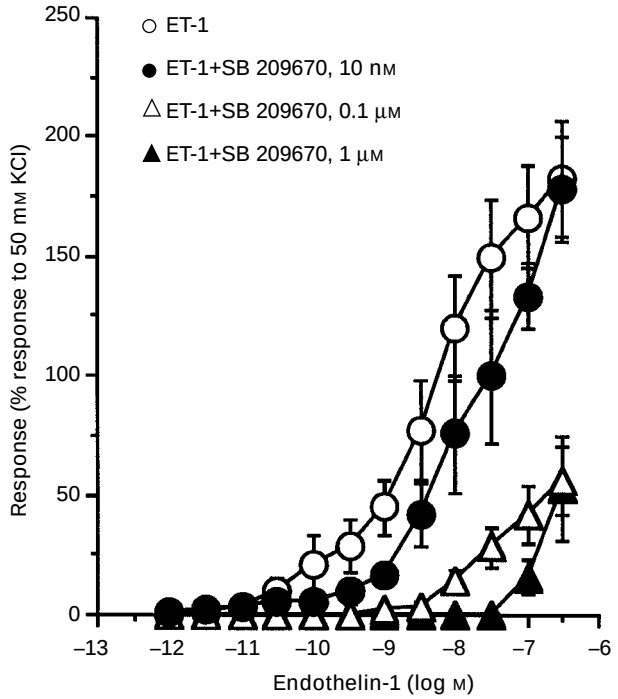

Figure 9 Effect of SB 209670 on responses to endothelin-1 (ET-1) in human pulmonary resistance arteries. ET-1 ($n=8$), ET-1 + 10 nM SB 209670 ($n=3$), ET-1 + 0.1 μM SB 209670 ($n=6$), and ET-1 + 1 μM SB 209670 ($n=6$). Data are expressed as percentage of reference contraction to 50 mM KCl in each vessel. n = number of animals. Each point represents the mean and vertical lines show s.e.mean.

However, in the present study, maximal responses to KCl were not increased and there was no increase in responses to SX6c. Whilst there was in increase in the maximum response to ET-1 in the chronically hypoxic rat vessels, there was no change in the potency of ET-1 or ET-3. In addition, FR139317 reversed the effect of hypoxia. This suggests that the increased response to ET-1 was due to an increase in ET_A-mediated vasoconstriction. Such an increase may occur if there was a decrease in endothelium-derived nitric oxide production, as has been demonstrated in lungs from chronically hypoxic rats (Adnot *et al*., 1991). However, we have previously demonstrated that endothelium-dependent relaxation is actually increased in the rat isolated pulmonary resistance artery (MacLean & McCulloch, 1998). It is more likely, therefore, that the increase in ET_A-mediated vasoconstriction observed in the chronically hypoxic rat vessels was due to an increase in receptor number. Indeed, an increase in the ET_A receptor gene has been observed in pulmonary arteries from chronically hypoxic rats (Li *et al*., 1994). This increase in ET_A-mediated response may contribute to the increase in pulmonary vascular resistance and pressures observed in chronically hypoxic rats. Indeed, it has been shown that the ET_A receptor antagonist BQ123 can reverse pulmonary hypertension in this model (Bonvallet *et al*., 1994; Dicarlo *et al*., 1995).

In control pulmonary resistance arteries, whilst responses to ET-1 were insensitive to the actions of the ET_A receptor antagonist FR139317, the non-peptide ET_A receptor antagonist BMS 182874 caused a small but significant increase in the potency of ET-1. There are three possible explanations for these observations: (i) in control rat pulmonary resistance artery, ET-1 does not activate typical ET_A receptors to mediate vasoconstriction, (ii) ET-1 activates an inhibitory ET_A receptor which negates any effect of typical ET_A receptor-mediated contraction, (iii) as in rabbit conduit pulmonary arteries, dual antagonism of ET_A and ET_B receptors is required to inhibit responses to ET-1 (Fukoroda *et al*., 1994). The order of potency for SX6c, ET-3 and ET-1 supports the concept that there is not a typical ET_A receptor present. However, the low potency of ET-1 could also be explained if ET-1 can act at an inhibitory receptor which opposed its vasoconstrictor action. There is certainly evidence that this may be the case with BMS 182874 increasing the potency of ET-1. Bosentan also increased the potency of ET-1 in the control rat vessels. Responses to SX6c were antagonized by bosentan in both control and chronically hypoxic preparations, with pK_b values of 5.84 and 6.11, respectively. Both SX6c and ET-3 were antagonized by BQ-788 (pK_b values, SX6c: 7.15 and 7.22; ET-3: 6.68 and 6.89 in control and chronically hypoxic rat vessels, respectively). In functional studies the pA_2 value for bosentan against ET_A receptor-mediated contraction was 7.3, compared with a value of 5.9 for ET_B-mediated constriction (Clozel *et al*., 1994). Bosentan might, therefore, have been exerting its effect on ET-1-induced responses in the rat pulmonary resistance arteries via blockade of vasodilator ET_B receptors located on the endothelium, or via the putative inhibitory ET_A receptor. We were unable to remove the endothelium of the rat pulmonary resistance arteries, to test this directly, without significantly damaging the fragile vascular smooth muscle. However, we could not demonstrate ET_B-mediated vasodilatation in this preparation (MacLean & McCulloch, 1998). In addition, the ET_B receptor antagonist BQ−788 had no effect on the potency of ET-1. We have also shown that inhibition of nitric oxide synthase has no effect on responses either to ET-1 or SX6c in pulmonary resistance arteries from control rats (MacLean & McCulloch, 1998). It is unlikely, therefore, that endothelial ET_B receptors are involved in the effect of bosentan on ET-1-induced responses in this preparation and it may indeed exert this potentiation through inhibition of an inhibitory ET_A receptor.

We considered the possibility that dual antagonism of ET_A and ET_B receptors is required to inhibit responses to ET-1. To address this, we tested the effects of bosentan and SB 209670 as well as looking at the effect of combining BQ-788 and BMS 182874 at a high concentration (10 μM). As discussed above, bosentan actually potentiated responses to ET-1 in control vessels and was without effect in the chronically hypoxic rat vessels. The combination of BQ−788 and BMS 182874 was without effect in the control vessels but caused a small but significant inhibition in the chronically hypoxic rat vessels, presumably because of the increased influence of the ET_A receptor in these vessels and/or the high concentration of BMS 182874. SB 209670 was unique in that it caused a significant inhibition of responses to ET-1 in vessels from both control and chronically hypoxic rats. Indeed it was the only antagonist tested which inhibited responses to ET-1. SB 209670 is extremely potent, having a pK_b value of $\sim$9.39 against ET-1 at an ET_A receptor site in the rat aorta, and a pK_b of 6.70 against ET-1 at an ET_B receptor site in the rabbit pulmonary artery (Ohlstein *et al*., 1994b). SB 209670 has pK_b values of 6.3, 6.1 and 6.8 against ET-1 in the rabbit bronchus, guinea-pig bronchus and rabbit pulmonary resistance artery, respectively (Hay *et al*., 1996; Hay & Luttmann, 1997; Docherty & MacLean, 1998). Here, we showed that it is relatively potent in the rat pulmonary resistance artery with a pK_b value of $\sim$7.4 in both control and chronically hypoxic rat preparations.

It is clear that the potency of antagonists can vary depending on both the species and on the endothelin agonist studied. BQ−

788 had a pK_b of 7.15 against SX6c which was significantly higher than that seen against ET-3 (6.68) yet did not inhibit responses to ET-1. A similar profile has been described in the rabbit large pulmonary artery, the rabbit pulmonary resistance artery and guinea-pig bronchus (Hay *et al*., 1996; Hay & Luttmann, 1997; Docherty & MacLean, 1998).

ET-1 was equipotent in the pulmonary resistance arteries from rat and man. We previously demonstrated, in human isolated pulmonary resistance arteries, that BQ-788 could inhibit responses to 1 pM – 1 nM ET-1, but concentrations greater than this were not inhibited by BQ-788. However, these higher concentrations of ET-1 were inhibited by 1 μM BMS 182874 which did not inhibit responses to 1 pM – 1 nM ET-1 (McCulloch *et al*., 1996). We showed here that SB 209670 was extremely potent in the human vessels over the entire CCRC and is the first antagonist, therefore, to demonstrate effective inhibition of responses to ET-1 in this preparation. Compounds with a similar pharmacological profile may, therefore, be extremely effective at lowering pulmonary vascular resistance in man.

ET-1 can exert an antiproliferative effect on pulmonary arterial smooth muscle and induce collagen remodelling in experimental pulmonary hypertension (Janakidevi *et al*., 1992; Mansoor *et al*., 1995). Both ET_A and ET_B receptors stimulate the mitogen-activated protein kinase cascade (Wang *et al*., 1994). However, in human pulmonary arterial and airway smooth muscle, the proliferative effect of ET-1 is thought to be mediated via the ET_A receptor (Zamora *et al*., 1993; Panettieri *et al*., 1996). Hence mixed endothelin receptor antagonists may exert both antiproliferative and antihypertensive effects on the pulmonary circulation in man. Clearly, the effectiveness of endothelin antagonists in man will depend upon their pharmacological profile.

The treatment of pulmonary hypertension with orally active endothelin antagonists has been investigated in the chronically hypoxic rat. Experimental approaches have varied but one group has studied the effect of both bosentan and the novel non-peptide ET_A antagonist A-127722 on both short- and long-term exposure to hypoxia (Chen *et al*., 1995; 1997). Whilst pulmonary pressures were not fully normalized and chronically A-127722 administration caused a significant elevation in systemic arterial pressure in one group, both antagonists prevented the pulmonary hypertensive effect of short-term hypoxia and attenuated the development of pulmonary hypertension. Whilst the effect on long-term hypoxia may be due to prevention of vascular remodelling, the effects on short-term hypoxia are probably mediated through blockade of ET_A receptors mediating vasoconstriction. This is compatible with our observations that ET_A receptor-mediated vasoconstriction is increased in the chronically hypoxic rat vessels. Hence, whilst ET_B-like receptors normally mediate vasoconstriction in rats, there is an influence of ET_A receptors in the chronically hypoxic, pulmonary hypertensive rat.

In conclusion, the present data indicate that vasoconstriction induced by ET-1, SX6c and ET-3 in the pulmonary resistance artery is mediated predominantly by activation of an ET_B receptor. However, the lack of effect of some of the endothelin antagonists used suggests that ET-1 induces contractions which involve stimulation of atypical ET_B receptors. In addition, the potentiating effects of other antagonists suggest the presence of an inhibitory ET_A receptor. In the chronically hypoxic, pulmonary hypertensive rat, responses to ET-1 are potentiated and this is mediated by the ET_A receptor. SB 209670 is unique in its ability to inhibit responses to ET-1 in human pulmonary resistance arteries and in control and pulmonary hypertensive rat pulmonary resistance arteries.

This study was funded by The Medical Research Council U.K., and The Wellcome Trust, U.K. The authors wish to thank Dr E. Ohlstein for donating SB 209670 for this study.

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(Received August 26, 1997
Revised December 5, 1997
Accepted January 14, 1998)